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Short communication

Contrasting effect of prey capture on jasmonate accumulation in two genera of aquatic carnivorous plants (*Aldrovanda*, *Utricularia*)

Jana Jakšová [a], Lubomír Adamec [b], Ivan Petřík [c], Ondřej Novák [c], Marek Šebela [d], Andrej Pavlovič [a,*]

[a] Department of Biophysics, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Šlechtitelů 27, CZ-783 71, Olomouc, Czech Republic
[b] Institute of Botany of the Czech Academy of Sciences, Department of Experimental and Functional Morphology, Dukelská135, CZ-379 82, Třeboň, Czech Republic
[c] Laboratory of Growth Regulators, Faculty of Science, Palacký University and Institute of Experimental Botany of the Czech Academy of Sciences, Šlechtitelů 27, CZ-783 71, Olomouc, Czech Republic
[d] Department of Biochemistry, Faculty of Science, and Centre of the Region Haná for Biotechnological and Agricultural Research, CATRIN, Palacký University, Šlechtitelů 27, CZ-783 71, Olomouc, Czech Republic

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ABSTRACT

Terrestrial carnivorous plants of genera *Drosera*, *Dionaea* and *Nepenthes* within the order Caryophyllales employ jasmonates for the induction of digestive processes in their traps. Here, we focused on two aquatic carnivorous plant genera with different trapping mechanism from distinct families and orders: *Aldrovanda* (Droseraceae, Caryophyllales) with snap-traps and *Utricularia* (Lentibulariaceae, Lamiales) with suction traps. Using phytohormone analyses and simple biotest, we asked whether the jasmonates are involved in the activation of carnivorous response similar to that known in traps of terrestrial genera of Droseraceae (*Drosera*, *Dionaea*). The results showed that *Utricularia*, in contrast with *Aldrovanda*, does not use jasmonates for activation of carnivorous response and is the second genus in Lamiales, which has not co-opted jasmonate signalling for botanical carnivory. On the other hand, the nLC-MS/MS analyses revealed that both genera secreted digestive fluid containing cysteine protease homologous to dionain although the mode of its regulation may differ. Whereas in *Utricularia* the cysteine protease is present constitutively in digestive fluid, it is induced by prey and exogenous application of jasmonic acid in *Aldrovanda*.

1. Introduction

Carnivorous plants represent an ecological group of ca. 800 species which capture, kill and digest animal prey in specialised modified leaves called traps, and use the absorbed nutrients for growth and development (Ellison and Adamec, 2018). It has been documented that three genera of carnivorous plants (*Dionaea*, *Drosera*, and *Nepenthes*) from order Caryophyllales use jasmonates (JAs) for activation of the digestive process in their traps. Jasmonic acid (JA), its isoleucine conjugate (JA-Ile) as well as their biosynthetic precursor, *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA), significantly accumulated in traps over time after experimental feeding and their exogenous application triggered the secretion of digestive enzymes and formation of digestive cavity (Escalante-Pérez et al., 2011; Nakamura et al., 2013; Libiaková et al., 2014; Yilamujiang et al., 2016; Krausko et al., 2017; Pavlovič et al., 2017, 2020). In non-carnivorous plants, JAs accumulate in response to pathogen or herbivore attack and activate plant defense reactions by transcriptional activation (Wasternack and Hause, 2013). It has been suggested that the jasmonate signalling pathway as well as digestive enzymes, which belong to pathogenesis-related proteins, have been co-opted by carnivorous plants from plant defense to prey digestion during evolution (Mithöfer, 2011; Pavlovič and Saganová, 2015; Bemm et al., 2016; Pavlovič and Mithöfer, 2019). The true bioactive compound JA-Ile binds to the CORONATINE INSENSITIVE1 (COI1) protein as a part of a co-receptor complex, mediates the ubiquitin-dependent degradation of JASMONATE ZIM-DOMAIN (JAZ) repressors, resulting in the activation of jasmonate-dependent gene expression (Thines et al., 2007; Fonseca et al., 2009; Sheard et al., 2010); in carnivorous plants, it initiates the expression of carnivory-related genes, mainly for nutrient transport and digestive enzymes (Bemm et al., 2016; Böhm et al., 2016;

* Corresponding author.
E-mail address: andrej.pavlovic@upol.cz (A. Pavlovič).

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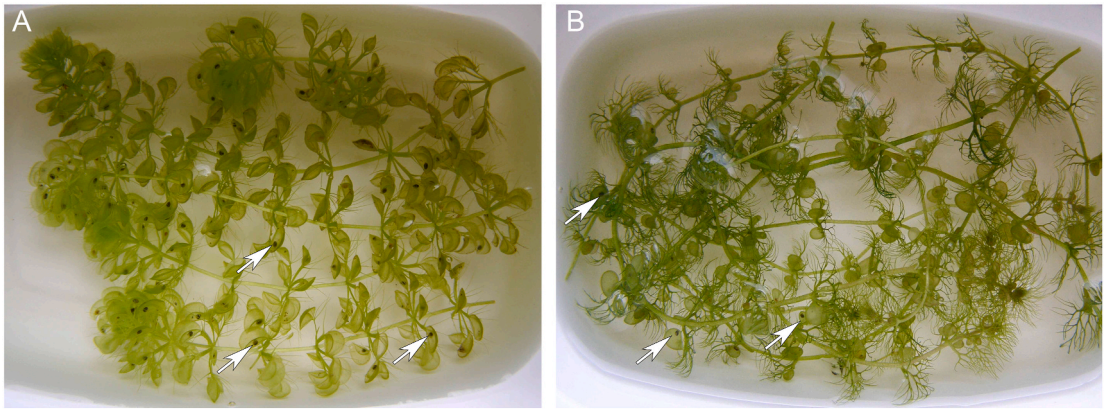

Fig. 1. Experimental setup. The plants were fed on aquatic prey for a short period. (A) *Aldrovanda vesiculosa*; (B) *Utricularia reflexa.* Arrows show the traps which successfully trapped prey and were used for analyses as fed traps.

Krausko et al., 2017; Pavlovič et al., 2017; Jakšová et al., 2020). However, all three genera mentioned above are closely related within the same order Caryophyllales. It has been recently reported that a carnivorous butterwort hybrid *Pinguicula* × *Tina* from the family Lentibulariaceae (order Lamiales) does not use jasmonate signalling for the induction of enzyme activities in response to prey capture, indicating that the jasmonate signalling is not a universal signalling pathway in all carnivorous plant genera (Kocáb et al., 2020).

In this study, we focused on two contrasting species of aquatic carnivorous plants. Aquatic carnivorous plants include monotypic *Aldrovanda vesiculosa* L. (Droseraceae, Caryophyllales) and about 60 submerged or amphibious species of *Utricularia* L. (Lentibulariaceae, Lamiales; Adamec, 2018). Although both genera of aquatic carnivores are ecologically very similar, the structures and functional principles of their motile traps are very different. *Aldrovanda* has 3–6 mm large snap-traps, reminiscent of those of the closely-related terrestrial Venus flytrap (*Dionaea muscipula*): two convex trap lobes are attached to a midrib and after mechanical irritation, generate action potentials and close within 14–50 ms (Iijima and Sibaoka, 1981, 1982; Poppinga et al., 2018; Westermeier et al., 2018, 2020). Although no digestive enzymes have been directly identified in the digestive fluid in *Aldrovanda* traps so far (Matušíková et al., 2018), exact ultrastructural studies on its digestive glands clearly revealed the stimulation mode of hydrolytic enzyme secretion after prey capture (Muravnik et al., 1995; Muravnik, 1996; Atsuzawa et al., 2020).

Suction traps of aquatic *Utricularia* species are 1–6 mm large, discoid hollow bladders with flexible side walls and contain a mobile sensitive trapdoor, hermetically sealing the trap. Continuous pumping of water out of the trap maintains a negative pressure inside the trap, which is the driving force for the prey capture (Adamec, 2018; Poppinga et al., 2016, 2018). The reset trap can open ('fire') very quickly after a mechanical stimulation or spontaneously after a critical negative pressure is reached (Adamec and Poppinga, 2016) and can repeatedly capture new prey every 30–60 min. Microbial commensal communities live inside the traps and function partly as digestion mutualists (Sirová et al., 2018a,b). Several classes of hydrolytic enzyme activities independent of prey capture have been reported from *Utricularia* traps (Sirová et al., 2003), but again no enzyme has been directly identified from the digestive fluid (Matušíková et al., 2018).

Analyses of phytohormones in traps were used in this study in two aquatic carnivores, *A. vesiculosa* and *Utricularia reflexa*, to find out whether their traps accumulate JAs in response to experimental feeding similar to terrestrial *Drosera*, *Dionaea* or *Nepenthes*. Simple biotests with JA, abscisic acid (ABA) and salicylic acid (SA) were conducted on the *Aldrovanda* trap closing reaction to reveal the possible regulatory effect of these metabolites on the activation of carnivory. In addition, LC-MS/MS analyses were used to identify new digestive enzymes in the digestive fluid.

2. Materials and methods

2.1. Plant material

Aldrovanda vesiculosa L. (origin from E Poland) and *Utricularia vulgaris* L. (from S Moravia, Czech Rep.) were grown outdoors in a 2 m^2 (volume 750 L) plastic container, while *Utricularia reflexa* Oliv. (from Botswana) and *Aldrovanda vesiculosa* (from N Australia) were grown indoors in naturally lit 3 L aquaria at the Institute of Botany in Třeboň (Czech Republic). A litter of robust *Carex* species was used as a substrate to mimic natural conditions. The water in both cultures was considered oligotrophic and moderately humic (for all details, see Sirová et al., 2003). Adult *Aldrovanda* plants were 12–20 cm long with traps 3–6 mm large, while *U. reflexa* plants were 20–30 cm long with traps 3–6 mm large and those of *U. vulgaris* were 60–80 cm long with traps 3.5–4.0 mm large. The use of *U. reflexa* was advantageous as this species has a small number of large traps.

2.2. Experimental design

Approximately 15 h before the feeding experiments, 25 robust plants of *A. vesiculosa* from the stock culture were shortened to 10 leaf whorls with mature traps (from the apex, shoot length 7—8 cm) and their branches were removed. Simultaneously, 10 plants of *U. reflexa* with large traps from the aquarium were shortened to10—15 leaf nodes (again from the apex, shoot length 6—8 cm) with mature traps; leaves bearing traps were excised from 3rd-12th mature leaf nodes (from the apex) of five *U. vulgaris* plants from the stock culture. The 15 h time period was chosen as sufficient based on the fact, that JA tissue level peaked 15–30 min after wounding in systemic tissue and then rapidly declined within 3 h to basal level (Koo et al., 2009). The plants or leaves were thoroughly washed in tap water and transferred to small plastic vessels with ca. 120 ml of filtered cultivation water taken from the outdoor plastic container. Five shoots (or seven excised leaves of *U. vulgaris*) were put in each small vessel. All traps with larger items of previously captured prey were removed. During ca. 15 h, most of the *Aldrovanda* traps were open and all *Utricularia* traps were reset and without air bubbles.

Relatively large zooplankton species (ostracods *Heterocypris incongruens* or diaptomids, copepods or daphnids) were added to the small vessels to feed the plants. After 20 min, at least 50% of *Aldrovanda* and 25% of *Utricularia* traps contained a prey (Fig. 1). Plants were then thoroughly washed in tap water again and put in small plastic vessels with ca. 200 ml of fresh filtered cultivation water without zooplankton. The vessels were transferred to a miniclimabox in continuous light (25

± 1 °C, fluorescent light 180 μmol m^{-2} s^{-1} PAR).

For phytohormone analyses, 2 and 24 h since prey addition, 12–20 traps (usually from 1 to 2 different shoots) with the captured prey, were cut as fast as possible for one sample and stored in ice-cold water during the manipulation. As a control, prey-free traps were sampled in parallel. The excised traps were then promptly (1–2 s) washed in tap water, blotted dry, weighed for fresh weight (FW), placed immediately in frozen 2 ml Eppendorf vials and stored in a freezer at −25 °C. Plant material was lyophilized immediately. Each sample had 1.5–3.7 mg of dry weight (DW). A DW/FW ratio (DW, 80 °C) was estimated in parallel material and each variant included 4–5 parallel samples from different plants. The zooplankton remained enclosed in the trap and it was not possible to remove it without causing trap damage. To estimate the hormone content in the prey, ostracods *Heterocypris incongruens* were also lyophilized for hormone analyses.

For protein determination experiment, the trap fluid was collected 24 h after feeding using the same set up from 240 fed traps of *A. vesiculosa* (unfed *A. vesiculosa* traps did not contain any digestive fluid and thus were not collected), 40 unfed and 50 fed traps of *U. reflexa*, and 26 unfed and 50 fed traps of *U. vulgaris*. The small glass capillary connected to peristaltic pump was inserted into *Utricularia* traps through the trap door or pierced through the *Aldrovanda* trap wall and digestive fluid was sampled. By this way, we obtained 130–400 μL of digestive fluid from different treatments.

2.3. Trap closing experiments

For studying the effect of JA, SA and ABA to induce trap closing, adult *Aldrovanda* plants from N Australia growing in indoor 3 L aquaria were used. Six short shoot segments containing 3rd–4th or 3rd–5th mature leaf whorls were cut, thoroughly washed in tap water, lightly blotted dry, and each segment was put in a transparent 30 mL plastic vial into 10 mL of the filtered cultivation water. The traps were left fully reopen for ca. 15 h. The individual phytohormones were added at a final concentration of 0.5 mM JA or 0.5 mM SA or 20 μM ABA at time 0 when each segment bore 15–23 open traps. These concentrations were chosen based on previous experiments on carnivorous plants, where they were found to be biologically active (Escalante-Perez et al., 2011; Nakamura et al., 2013; Buch et al., 2015; Krausko et al., 2017; Pavlovič et al., 2017). The segments were exposed under the same conditions as above. Closed traps were counted after 20, 40, 55, 120 and 180 min and observed for additional 48 h. Segments without added phytohormones were used as a control. Digestive fluid from JA-induced closed *Aldrovanda* traps was also collected as described above for MS analyses 24 h after the application of 0.5 mM JA. Due to spontaneous firing of *Utricularia* traps several times per day (Adamec, 2011; Vincent et al., 2011), it was pointless to repeat the same experiment with *U. reflexa* traps.

2.4. Quantification of phytohormones

The phytohormones were quantified in *A. vesiculosa* and *U. reflexa*. Quantification of phytohormones was performed according to the method described by Floková et al. (2014); the extraction process was modified for a small amount of dry plant tissue. One mL of ice-cold 10% MeOH/H_2O (v/v), internal standards and four small metallic beads were added to the dry biomass. Dry plant material was homogenized using a MM 301 vibration mill (Retsch GmbH & Co. KG, Haan, Germany) at a frequency of 27 Hz for 5 min. The samples were incubated at 4 °C by shaking using a laboratory rotator for 30 min and centrifuged (20,000 rpm, 4 °C, 15 min). The supernatant was transferred into a new Eppendorf vial, the volume was measured and the extract of most of the samples was subdivided (as dependent on the FW) into two aliquots. At the end, each sample contained stable isotope-labelled standards as follows: 10 pmol of [$^2H_6$]JA, [$^2H_5$]OPDA, [$^2H_6$]ABA, [$^{13}C_6$]IAA, 0.1 pmol of [$^2H_2$]JA-Ile and 20 pmol of [$^2H_4$]SA (all from Olchemim Ltd., Czech Republic) to validate the LC-MS/MS method. The extracts were purified using Oasis® HLB columns (30 mg mL^{-1}, Waters, Milford, MA, USA) and hormones were eluted with 80% methanol. The eluent was evaporated to dryness under a stream of nitrogen. Phytohormone levels were determined by ultra-high performance liquid chromatography-electrospray tandem mass spectrometry (UHPLC-MS/MS) using an Acquity UPLC I-Class System (Waters, Milford, MA, USA) equipped with an Acquity UPLC CSH C18 column (100 × 2.1 mm; 1.7 μm; Waters) coupled to a triple quadrupole mass spectrometer Xevo TQ-S MS (Waters MS Technologies, Manchester, UK).

2.5. Protein identification analysis

Protein concentration was determined by the bicinchoninic acid assay (Smith et al., 1985). Sample of fed *A. vesiculosa* showed the highest value of 2.1 mg ml^{-1} protein, sample of fed *U. vulgaris* contained 0.1 mg ml^{-1}; the others had even lower content. All samples were subjected to SDS-PAGE (Laemmli, 1970) in a 12% T/2.7% C resolving gel and 4% T/2.7% C stacking gel; 30-μL aliquots in Laemmli sample buffer were loaded per well onto an 8 × 7 cm minigel, 1-mm thick. Coomassie-stained protein bands were excised from the gel slab, which was followed by an in-gel digestion step (Shevchenko et al., 2006). The resulting digests were purified on ZipTip C18 pipette tips (Merck-Millipore, Ireland) and the recovered desalted peptides separated by nanoflow liquid chromatography coupled to electrospray ion trap tandem mass spectrometry (nLC-ESI-IT-MS/MS) on an amaZon speed ETD instrument (Bruker Daltonik, Bremen, Germany) as already described (Panáček et al., 2018).

MGF formatted nLC-ESI-IT-MS/MS data files were searched against Caryophyllales and Lamiales protein sequences downloaded from the NCBI Protein database (https://www.ncbi.nlm.nih.gov/protein/) in March 2021, and supplemented with CRAP contaminants database (https://www.thegpm.org/crap/), using PEAKS X software (Bioinformatics Solutions, Inc., Waterloo, ON, Canada). The data were also searched against the reviewed database Swiss-Prot (release 2021_01; https://www.uniprot.org/downloads), taxonomy Vidiriplantae. Parameters of the searches were as follows: monoisotopic masses; error tolerance for precursor mass of 50 ppm; error tolerance for fragment ions of 0.5 Da; semispecific trypsin digestion mode; up to three missed cleavages; carbamidomethylation of cysteine as a fixed modification; oxidation of methionine and acetylation of protein N-terminus as variable modifications; three maximum variable modifications per peptide.

Gel-based protein identification after the induction by 0.5 mM JA in *Aldrovanda* was not successful because of a low protein content. To overcome this, a 200-μl aliquot of the digestive fluid was dried out in a vacuum centrifuge. The solid residue was dissolved in 50 μl of 100 mM NH_4HCO_3 and alkalified by adding 1 μl of 25% (v/v) ammonia. Disulfides were reduced by adding 2 μl of 100 mM dithiothreitol (DTT) in 100 mM NH_4HCO_3 and incubating at 37 °C for 30 min. After cooling down, 5 μl of 100 mM iodoacetamide in 100 mM NH_4HCO_3 were added and the mixture incubated at 23 °C in the dark for 20 min. Then 2.5 μl of 100 mM DTT in 100 mM NH_4HCO_3 were added for quenching the unreacted alkylating reagent. After 20 min, the solution was adjusted to a total volume of 300 μl by 50 mM NH_4HCO_3. Digestion was initiated by 3 μL of SOLu trypsin (Merck, Germany) and proceeded at 37 °C for 24 h. The digest was evaporated to dryness in vacuum centrifuge and reconstituted in 10 μl of 0.1% (v/v) trifluoroacetic acid. Peptides from the digest were purified using a ZipTip C18 pipette tip. The procedure of protein identification was based on nLC coupled via an eluate-spotting device to MS/MS on a MALDI-TOF/TOF instrument as already described (Petrovská et al., 2014). MS/MS data were processed by database searches using PEAKS X as above (plus manually evaluated using flexAnalysis 3.4 and BioTools 3.2 by Bruker Daltonik); glutamine/asparagine deamidation was an additionally considered variable modification.

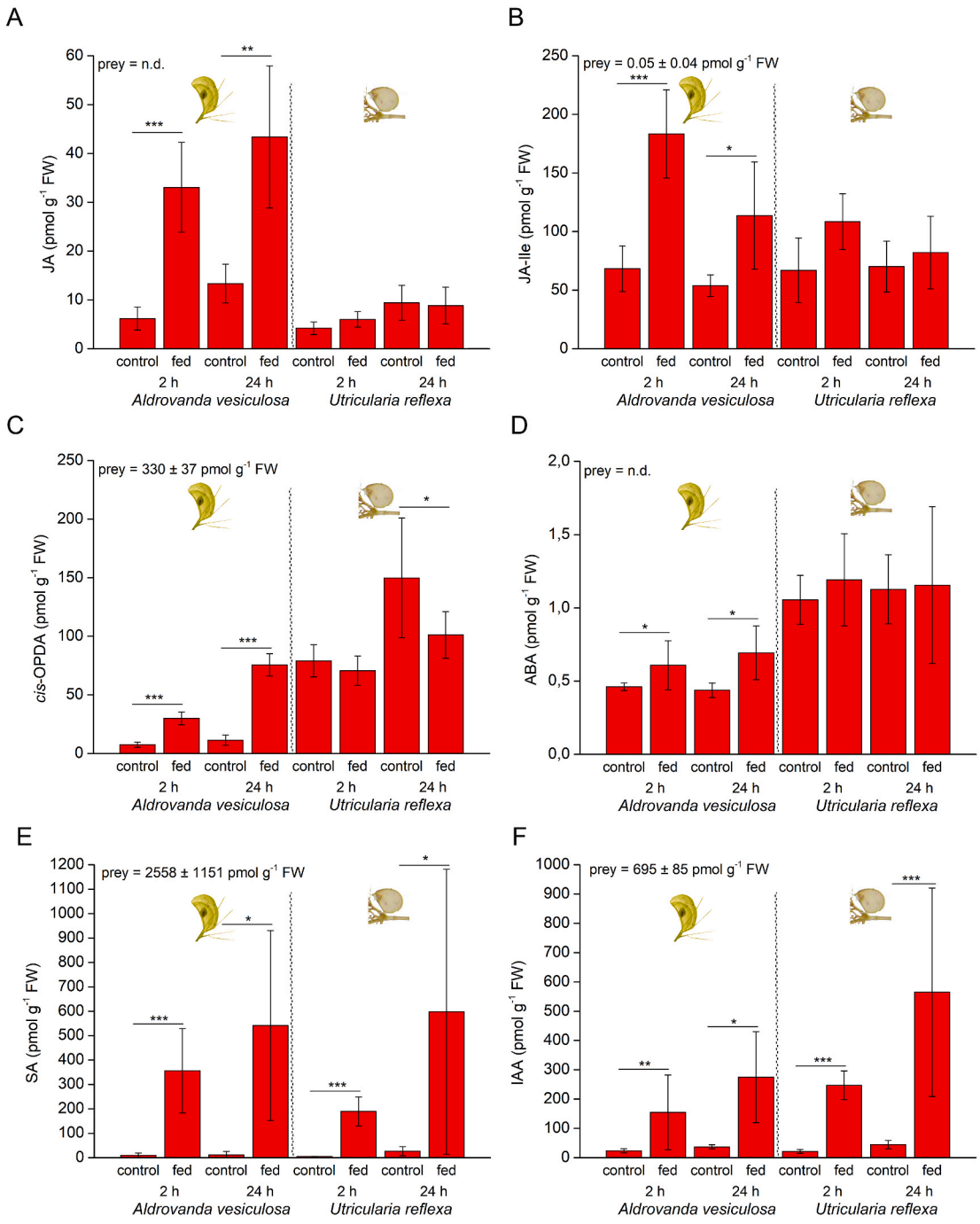

Fig. 2. Phytohormone trap tissue content. (A) Jasmonic acid, (B) jasmonic acid isoleucine conjugate, (C) *cis*-12-oxophytodienoic acid, (D) abscisic acid, (E) salicylic acid, (F) 3-indolacetic acid. Phytohormone content in the prey is shown in upper left corner. Means ± S.D., n = 4–10, n.d. – not determined (below detection limit).

2.6. Statistical analyses

Means ± SD intervals are shown. The statistically significant differences between fed variants and unfed controls were tested by the Student *t*-test. If non-homogeneity was present, the Welch *t*-test was used (Microsoft Excel).

3. Results and discussion

Morphological and physiological features and growth strategies of aquatic carnivorous plants are quite dissimilar from those of terrestrial ones (Adamec, 2018). The submerged aquatic or amphibious species of *Aldrovanda* and *Utricularia* are strictly rootless vascular plants that grow in dystrophic, barren waters. Here, we investigated whether the JAs accumulated in response to feeding in the traps of two distantly-related aquatic species of carnivorous plants, *Aldrovanda vesiculosa* and *Utricularia reflexa,* are similar to that in traps of some terrestrial species.

Aldrovanda vesiculosa accumulated significantly increased levels of JA, JA-Ile, *cis*-OPDA and ABA in trap tissues after both 2 and 24 h following experimental feeding on zooplankton. In contrast, *Utricularia reflexa* did not accumulate significant levels of these phytohormones in trap tissues after experimental feeding (Fig. 2A–D). The significant increase in the level of SA and indole-3-acetic acid (IAA) in fed traps of both species can be attributed to the high content of these phytohormones in the applied zooplankton, which remained enclosed in the traps and was thus analyzed together with trap tissue (Fig. 2E and F). To verify

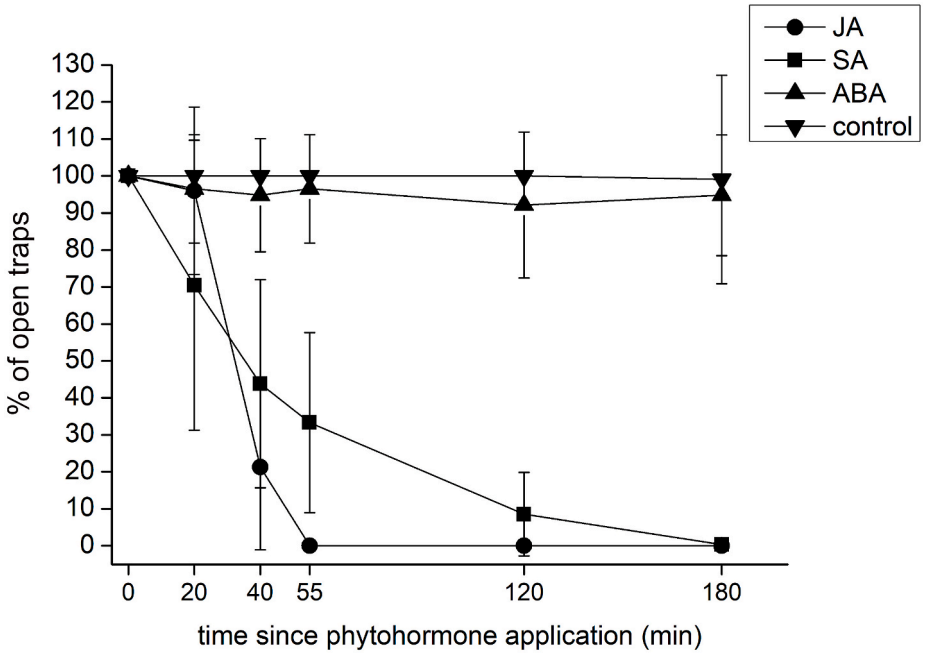

Fig. 3. Trap closing response in *Aldrovanda vesiculosa*. The phytohormones (0.5 mM JA, 0.5 mM SA, 20 μM ABA) were applied at time point 0 min, and numbers of closed traps were counted at regular time interval. Means ± S.D., n = 6 shoot segments.

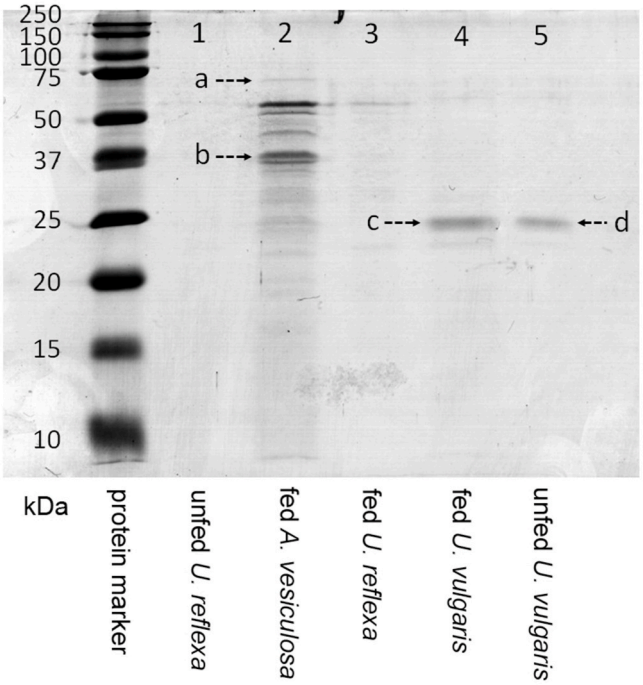

Fig. 4. Sodium dodecylsulfate polyacrylamide gel electrophoresis of digestive fluids. Protein samples were separated using a 12% resolving gel and 4% stacking gel. The resolving gel was stained afterwards by Coomassie Brilliant Blue G-250. The separation lanes show, from the left, a protein marker with the indicated molecular mass values of its components, and samples of digestive fluid from: 1 - unfed *U. reflexa*, 2 - fed *A. vesiculosa*, 3 – fed *U. reflexa*, 4 – fed *U. vulgaris*, 5 – unfed *U. vulgaris*. Arrows indicate the visualized proteins bands (2-a, 2-b, 4-c, 5-d), which were confirmed to contain hydrolytic enzymes as identified by nLC-ESI-IT-MS/MS.

the physiological effect of JAs; JA, SA and ABA were added into the cultivation water to a final concentration of 0.5 mM, 0.5 mM and 20 μM, respectively, and we observed the trap closing reaction. In the trap closing experiments on applied phytohormones, all *Aldrovanda* traps were closed after the application of JA after 55 min (Fig. 3) and stayed closed for a period of 48 h. Moreover, traps immersed in the JA medium for 10 h also stayed closed in the fresh medium without JA for the next 84 h (data not shown). The trap closing reaction was also induced by SA (Fig. 3), but after 44 h, all traps were damaged by SA and most probably were dead. After transfer into the fresh medium without SA, the traps only slightly re-opened but did not react to mechanical stimuli, indicating a pharmacologically damaging effect of SA. ABA was not able to induce the trap closing reaction at all (Fig. 3) and the traps fully closed upon mechanical stimulation after 44 h of the ABA treatment.

Despite its aquatic lifestyle, *Aldrovanda* accumulates JAs in response to prey capture and responds also to their exogenous application in a way similar to the terrestrial carnivorous members of Droseraceae, *Drosera capensis* and *Dionaea muscipula* (Nakamura et al., 2013; Libiaková et al., 2014; Krausko et al., 2017; Pavlovič et al., 2017). As *Aldrovanda* generates APs in response to mechanical stimulation (Iijima and Sibaoka, 1981, 1982) like its closest relative *Dionaea* (Hodick and Sievers, 1988), the downstream sequence of signalling events is probably similar and involves Ca^{2+} induced JAs accumulation as is well-known in non-carnivorous plants (Toyota et al., 2018; Farmer et al., 2020; Suda et al., 2020). In *Dionaea*, accumulated JAs activated the expression of digestive enzymes including cysteine protease dionain and VF chitinase I (Libiaková et al., 2014; Böhm et al., 2016; Pavlovič et al., 2017) and we found similar enzyme in *Aldrovanda.* Based on our nLC-MS/MS analysis and homology search, we identified a cysteine protease dionain3 from *Dionaea muscipula* (accession no. gi|794462956 assigned by a single peptide NSWGTSWGENGYIR to the band 2-b in Fig. 4) in the digestive fluid collected from fed *Aldrovanda* traps (we called it aldrovandain). Digestive fluid collected from hermetically closed traps induced by exogenously added 0.5 mM JA also contained cysteine protease in *Aldrovanda* (identification based on the same sequence as above). Therefore the mechanism of enzyme synthesis is the same as in *Dionaea*. Moreover, putative nucleotide pyrophosphatase/-phosphodiesterase from *Nepenthes mirabilis* (accession no. gi|1002635122) was assigned by a single peptide FLAFGDMGK to the protein band 2-a in Fig. 4. A recent genomic study has revealed that *Aldrovanda*, *Dionaea* and *Drosera* have significantly expanded gene

families related to jasmonate signalling (Palfalvi et al., 2020). Thus, our phytohormone analyses in *Aldrovanda*, *Drosera* (Krausko et al., 2017) and *Dionaea* (Libiaková et al., 2014; Pavlovič et al., 2017, 2020) are important physiological evidence to genome studies supporting the hypothesis that jasmonate signalling was co-opted for carnivory likely already in a common ancestor of the Droseraceae (Palfalvi et al., 2020). Based on the molecular evidence, it was proposed that the snap-traps of *Aldrovanda* and *Dionaea* were derived from a common terrestrial ancestor that had flypaper-traps (Cameron et al., 2002) which co-opted JA signalling from plant defense (Palfalvi et al., 2020). Later, probably terrestrial ancestor of *Aldrovanda* was becoming adapted to permanently aquatic lifestyle (Cameron et al., 2002).

On the other hand, the aquatic *Utricularia reflexa*, which uses different trapping mechanism and is not related to Droseraceae, does not accumulate significant amount of JAs in response to feeding. This is in accordance with genome analysis of *U. gibba*, where, in contrast with Droseraceae, gene families related to jasmonate signalling are not significantly expanded (Ibarra-Laclette et al., 2013; Carretero-Paulet et al., 2015; Lan et al., 2017). The findings that enzyme activity in *Utricularia* bladders is independent of prey capture and is rather constitutive (Sirová et al., 2003) question the necessity to possess a JA-inducible system for enzyme secretion. In the trap fluids of *Utricularia*, there are many species of living bacteria, algae, fungi and protozoa and it has been suggested that *Utricularia* are rather more 'farmers' than 'hunters' (Sirová et al., 2018a,b). Even more, putative losses of the defense response genes in *U. gibba* are apparent (Renner et al., 2018). Microbiome organisms certainly contribute to hydrolytic activities in the digestive fluid, but the plants also secrete their own digestive enzymes (Sirová et al., 2003). Using our nLC-MS/MS analyses and homology search we were unsuccessful to identify any secreted enzyme in *U. reflexa*. Therefore, we used another *Utricularia* species and found that the major bands in samples from fed and unfed *U. vulgaris* digestive fluid, i.e. 4-c and 5-d (Fig. 4), respectively, were assigned by a single peptide DQGQCGSCWAF to dionain 4 from *Dionaea muscipul*a (accession no. gi|1114672835) or cysteine protease from *Spinacia oleracea* (accession no. gi|222425026). Indeed, the *U. gibba* genome reveals large expansions of cysteine protease gene family which are predominantly expressed in trap tissue (Lan et al., 2017). It seems that different evolutionary lineages of carnivorous plants co-opted similar digestive enzymes with convergent amino acid changes (Fukushima et al., 2017), but the mode of their regulation may differ. Moreover, there is an obvious difference in the molecular mass (Fig. 4). Additional protein identifications in samples including abundant plant intracellular proteins such as actin, glyceraldehyde-3-phosphate dehydrogenase, fructose bisphosphate aldolase, triosephosphate isomerase, calmodulin, histones, and ubiquitin were considered contaminants arising from a mechanical injury of plants during the sample collection process.

In addition to our previous study on *Pinguicula* (Kocáb et al., 2020), *Utricularia* is the second genus of carnivorous plant in the order Lamiales, which has not co-opted jasmonate signalling for botanical carnivory. On the other hand, activation of digestive process in aquatic *Aldrovanda* is similar to terrestrial *Dionaea* and both rely on jasmonates. Interestingly, both genera of aquatic carnivorous plants used cysteine protease homologous to dionain for prey digestion, which is prey and JA-induced in *Aldrovanda* and constitutively present in *Utricularia*. Thus, although the similar digestive enzymes were co-opted for botanical carnivory, the mode of their regulation may differ in different taxa. How are the carnivory-related processes activated by prey in other genera of CPs remains to be investigated.

Author's contribution

AP and LA designed the experiments; JJ and LA collected samples and did the biotest; JJ, IP and ON analyzed phytohormone tissue level; MS performed nLC-MS/MS; JJ and AP wrote the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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